Cross-species hybridization of microarrays for studying tumor transcriptome of brain metastasis

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Although the importance of the cellular microenvironment (soil) during invasion and metastasis of cancer cells (seed) has been well-recognized, technical challenges have limited the ability to assess the influence of the microenvironment on cancer cells at the molecular level. Here, we show that an experimental strategy, competitive cross-species hybridization of microarray experiments, can characterize the influence of different microenvironments on cancer cells by independently extracting gene expression data of cancer and host cells when human cancer cells were xenografted into different organ sites of immunocompromised mice. Surprisingly, the analysis of gene expression data showed that the brain microenvironment induces complete reprogramming of metastasized cancer cells, resulting in a gain of neuronal cell characteristics and mimicking neurogenesis during development. We also show that epigenetic changes coincide with transcriptional reprogramming in cancer cells. These observations provide proof of principle for competitive cross-species hybridization of microarray experiments to characterize the effect of the microenvironment on tumor cell behavior.

genomics | tumor microenvironment | stromal cells | DNA methylation

During tumorgenesis, the changes in the local microenvironment play major roles in each step in metastasis from dissemination to colonization of distant organ sites (1–4). Therefore, inhibition of the cross-talk between invasive cancer cells and stromal cells might delay or completely block tumor invasion and subsequent colonization of cancer cells. However, a lack of understanding of the bidirectional communication between cancer cells and stromal cells at the molecular level hampers the ability to develop and implement useful targeted therapies.

Despite the potential to obtain stromal- and cancer-specific signatures using laser-captured microdissection (5), segregating cancer cells from stromal cells and vice versa remain a time-consuming and labor-intensive procedure that is technically challenging. There is also a high risk of failure because of instability of RNA during cell isolation as well as the potential systemic bias of gene expression during amplification of partially degraded RNA (6, 7). Thus, there is an urgent need for the development of new experimental strategies that can delineate cancer cell-specific gene expression signatures from stromal cell signatures or vice versa.

In vivo xenografting of human cancer cells to different mouse organs has the potential to elucidate the influence of the tumor microenvironment on gene expression in cancer cells by mimicking the conditions that human metastases undergo at different organ sites. Characterization of these in vivo models might provide clues for the specific role of each unique tumor microenvironment in determining the propensity of particular tumors to metastasize to different sites as well as the sensitivity and resistance to therapy at different sites. In the current study, we have devised and performed proof of concept studies for an experimental strategy, competitive cross-species hybridization of microarray experiment (CHME), that can simultaneously acquire gene expression patterns of cancer cells and host cells from a single xenograft tissue by hybridizing an unmanipulated population of RNAs with human or mouse microarrays. This approach is based on the concept that, in xenograft tumor tissues, all cancer cells are solely human, although all host cells are of murine origin. When CHME strategy was applied to in vivo metastasis models, it showed that CHME can reliably separate complex gene expression patterns from mixed cell populations in a single xenografted tumor mass and elucidate functional consequences of tumor environment interactions.

Results

Competitive Cross-Species Hybridization. To selectively profile tumor and stromal cells in CHME, we used Illumina's Sentrix Beadchip microarrays, because they are based on long oligomer DNA probes (50-mer) and use highly stringent hybridization conditions (58 °C) during microarray experiments. To test species specificity of DNA probes in Illumina's human and mouse microarrays, we first conducted BLAST nucleotide searches of mouse and human probe sequences on the Sentrix Beadchip microarrays of the human and mouse mRNA databases, respectively (8). The vast majority of human and mouse probes showed low bit scores on cross-species BLAST nucleotide. Indeed, more than 95% of human and mouse probes showed bit scores less than 40 and 56, respectively (Fig. S1*A*), indicating high species specificity of selected probe sequences.

To test the validity of the experimental strategy, we next carried out cross-species hybridization of microarray experiments with human (MDA-MB-231) and mouse RNA (immortalized astrocytes). Labeled cRNAs of human MDA-MB-231 and mouse astrocytes were hybridized on both human and mouse microarrays slides (Fig. S2*A*). To test the sensitivity of competitive hybridization, five different mixtures (90% human and 10% mouse, 70% human and 30% mouse, 50% human and 50% mouse, 30% human and 70% mouse, and 10% human and 90% mouse) were applied to both human and mouse microarrays. Strikingly, signals for more than 95% of the probes in human microarrays were dependent on the amount of labeled human cRNA present in the

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hybridization (Fig. S1*B*), indicating that the presence of mouse cRNAs (10%, 30%, 50%, 70%, or even 90%) did not compete or significantly inhibit hybridization between human cRNA and human oligo probes in the microarrays. Likewise, hybridization of the mixtures on mouse microarrays showed similar patterns of signal intensity (Fig. S1*C*), indicating that the presence of human cDNA has minimal effect on hybridization of murine DNA to the mouse microarray as well. These results strongly suggest that the optimized experimental conditions with the Sentrix Beadchip microarrays provide sufficient specificity to discriminate expression of mouse and human genes when mRNA from both species is mixed together and that they maintain the necessary sensitivity to detect the expression of species-specific genes even when they only represent 10% of the mRNA mixture.

Comparison of Gene Expression Profiles of Xenografted Tumors. To test the feasibility of CHME to detect signals from human or murine cells in in vivo conditions, we next carried out experiments with xenografted human cancer cells (MDA-MB231Br3, PC14Br4, KM12M, and A375SM) in mice. To examine the influence of different microenvironments on the transcriptional profiles of the cancer cells, cancer cells were xenografted in three different murine organ sites (brain, skin, and orthotopic tumor sites) as described in Fig. S2*B*. RNA from each xenografted tissue was labeled, split into two parts, and hybridized to human and mouse microarrays separately. Hierarchical clustering analysis was first applied to gene expression data from a human microarray representing cancer cells. As expected, the four different cancer cell lines xenografted orthotopically or s.c. were clustered based on the identity of the cancer cell line independent of the location of the transplant with few exceptions (Fig. 1*A*). For example, gene expression patterns of s.c.-xenografted breast cancer MDA-MB-231Br3 were very similar to MDA-MB-231Br3 orthotopically xenografted to the mammary fat pat, indicating that the gene expression program reflecting the origin of cancer cells remains relatively unchanged even when they were grown in two different peripheral microenvironments (i.e., fat pad and skin in MDA-MB-231).

Surprisingly, with the exception of 2 PC14Br4 tumors and 1 KM12M tumor, 21 tumors from the four different cancer cell lines growing in the brain showed substantially different gene expression patterns to the same tumors at either the s.c. or orthotopic site. The overall expression patterns lacked the cell line-specific gene expression signature reflecting the cellular origin of the cancer cells, indicating that cancer cells growing in the brain no longer maintained their cell line-specific gene expression program. Because distinct gene expression patterns in the cancer cells growing in the brain might be caused by the contamination of human RNA with abundant mouse brain RNA, we included mixtures of RNA from mouse brain and PC14Br4-cultured cells in microarray experiments as control. Gene expression patterns of the mixed RNA were highly similar to PC14Br4 xenografted to lung or skin (sample indicated by asterisk in Fig. 1*A*), strongly indicating that presence of mouse brain RNA has a limited influence during hybridization on human microarrays.

Species-specific detection of gene expression signals during hybridization was also evident when hierarchical clustering analysis was applied to gene expression data from mouse microarray (noncancer cell data). The brain-xenografted tumors clustered together with normal mouse brain samples and were well-separated from the rest of tissues (Fig. 1*B*). Moreover, mixed RNA (containing normal mouse brain RNA and PC14Br4 RNA) clustered with brain tissues, indicating that gene expression patterns from mouse microarrays were largely dependent on mouse RNA in the xenografted tissues. To further validate species-specific detection of gene expression and cancer cell-specific expression of brain-induced genes in brain-xenografted

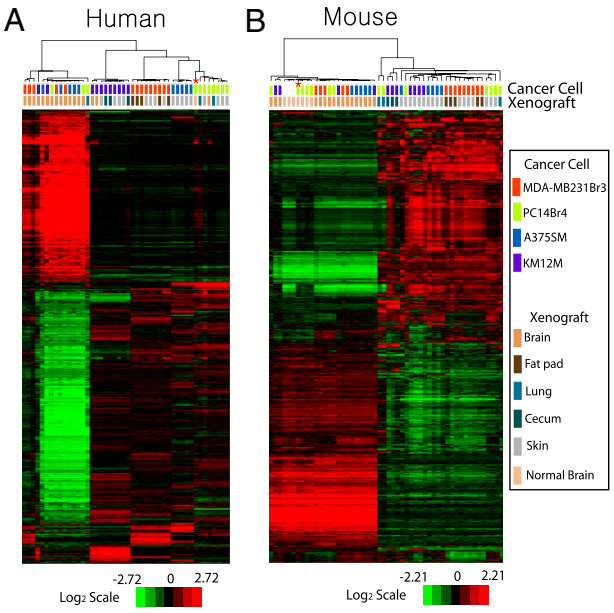

Fig. 1. Gene expression patterns of xenografted tissues. (*A*) Hierarchical clustering analysis of gene expression data from human microarray experiments with xenografted tumors. Genes with expression variance within the 25th percentile and having at least a 1.5-fold difference in at least 20% of samples relative to the median value across all samples were selected for clustering analysis (11,156 gene features). The data are presented in matrix format, in which rows represent individual genes and columns represent each tissue. Each cell in the matrix represents the expression level of a gene feature in an individual tissue. The color red or green in cells reflects relative high or low expression levels, respectively, which is indicated in the scale bar (log2-transformed scale). To test the influence of mouse RNA during hybridization, mouse brain RNAs were mixed with the RNAs from PC14Br cells in cell culture before microarray experiments (highlighted with asterisk). (*B*) Hierarchical clustering analysis of gene expression data from mouse microarray experiments with the same xenografted tumor tissues used in human microarray experiments. Expression data of 10,160 gene features were used for this analysis after applying variance filtration. Gene expression data generated with mixed RNA were also highlighted. The first and second letters of the sample identification code in the dendrogram represent cancer cell lines and xenografted organ sites, respectively. A, A375SM; K, KM12M; M, MDA-MB231Br3; P, PC14Br4 for cell lines. B, brain; C, cecum; F, fat pad; L, lung; S, skin for organ sites.

tumor, we carried out immunohistochemical (IHC) staining of formalin-fixed paraffin-embedded (FFPE) tissues from xenografted tumors. Our gene expression data showed that expression of *CCL27*, *KIF1A*, and *NFIB* is highly specific to MDA-MB-231 cells xenografted to brain (Fig. S3*A*). IHC staining of FFPE tissues with specific antibodies was in good agreement with gene expression data. Although expression of three proteins was well-detected in brain-xenografted human cancer cells, expression of them in mouse brain (with xenografted human cancer cells in neighbor) and human cancer cells xenografted in fat pad was barely detectable (Fig. S3*B*). To further show that induced expression of CCL27 in brain-xenografted human cancer cells is not caused by staining of mouse brain cells, FFPE tissues of brain-xenografted MDA-MB-231 were double-stained with anti-CCL27 and anti-GFAP, which is a specific marker for astrocytes in brain, using immunofluorescence techniques. Expression of CCL27 is mutually exclusive to expression of GFAP in brain-xenografted tumors (Fig. S3*C*), indicating that CCL27 is not expressed in mouse brain cells. Thus, these data clearly showed that expression of these proteins was only specific to human cancer cells growing in the brain. In an additional subset analysis, we selected genes with expression patterns that very significantly

changed ($P < 1.0 \times 10^{-4}$) in brain-xenografted cancer cells compared with fat pad-xenografted cancer cells and asked whether their expression patterns are highly similar to those patterns of mouse brain. Although a small fraction of gene expression patterns was similar to mouse brain, the majority of them was very unique to brain-xenografted cancer cells (Fig. S4*A*). This analysis also strongly suggested that altered gene expression in brain-xenografted cancer cells is not caused by cross-hybridization of mouse mRNA to human probes during microarray experiments.

We next assessed differences in gene expression patterns among the four cancer cell lines when growing in different organ sites by applying ANOVA. Expression of a large number of genes (4,213 genes) was significantly different ($P < 1.0 \times 10^{-7}$) among s.c.-xenografted tissues, reflecting inherent differences in the transcriptomic program among the four cancer cell lines. However, the same threshold cutoff ($P < 1.0 \times 10^{-7}$) only identified 21 RNAs as significantly different in the tumor lines growing in the brain (Fig. S4 *B* and *C*), suggesting that the brain microenvironment not only overrides the cell line-specific transcriptome of cancer cells but also orchestrates a drastic reprogramming of the cancer transcriptome to engender a similar gene expression pattern in the different cell lines and cell lineages.

Acquisition of Neuronal Cell Characteristics of Cancer Cells in the Brain Microenvironment. To uncover the characteristics underlying the transcriptional pattern present in cancer cells in the brain microenvironment, we applied gene set analysis from the transcriptional profiles of A375SM cells growing in brain using Ingenuity Pathway Analysis. Surprisingly, the most enriched gene sets among the up-regulated genes in A375SM growing in brain were those sets involved in neurological signaling. For example, genes implicated in neuropathic pain signaling, synaptic long-term potentiation, axonal guidance signaling, and glutamate receptor signaling are significantly enriched (Fig. S5 *A–C*). *SNAP25* (261-fold increase), *SNAP91* (254-fold increase), and *BSN* (181-fold increase) components of the neurotransmitter complex that are uniquely expressed in neuronal cells were highly up-regulated in tumor cells growing in brain (Fig. S5*D*) (9–11), strongly suggesting that cancer cells in the brain microenvironment acquire characteristics of neuronal lineage cells. In support of the contention that cancer cells growing in the brain microenvironment express genes typical of neurons, the expression of these genes in the three other cancer cell lines growing in the brain showed marked overexpression of each of the candidates without any detectable expression when growing in orthotopic or s.c. sites (Fig. S5*E*). In contrast, the most significantly enriched gene sets among down-regulated genes in tumor cells growing in the brain related to energy-generating metabolic pathways (Fig. S5*F*). Genes involved in oxidative phosphorylation (Fig. S5*G*), mitochondrial function, glycolysis/gluconeogenesis, and the pentose phosphate pathway were significantly down-regulated in cancer cells growing in brain.

Epigenetic Changes Driven by the Brain Microenvironment. The characteristics of altered gene expression patterns in tumor cells growing in the brain, the large number of differentially expressed genes, and the acquisition of neuronal development lineage-specific markers in all four cancer cell lines seemed to reflect the differentiation or transdifferentiation of cells that occurs during development rather than the progression of the cancer cells into a more undifferentiated state. Because epigenetic events regulate the differentiation potential and fate specification of stem or progenitor cells (12), we next sought to investigate epigenetic changes in the cancer cell lines growing in the brain.

We collected genome-wide methylation profiles of gene promoters from the 24 xenografted tissues used for gene expression profiling analysis. Unsupervised hierarchical clustering analysis revealed that most of the tumors growing in the brain shared a similar methylation profile, whereas orthotopically xenografted tissues showed characteristic methylation patterns reflecting the origin of the cancer cell line, which was evidenced by distinctive methylation patterns in each cell line (Fig. 2*A*). Interestingly, three brain xenografts (two KM12M and one PC14Br4 xenografts) that grouped with orthotopically xenografted tissues in gene expression profiling (Fig. 1*A*) also clustered together with orthotopically xenografted tissues on methylation analysis. Moreover, an orthotopically xenografted PC14Br4 sample previously grouped with tumor cells growing in brain based on CHME clustered together with cells growing in brain based on methylation profiling. These results strongly indicate that promoter methylation profile reflects and potentially contributes to the gene expression profile.

When direct association between methylation status of promoters and expression of corresponding genes was estimated by Pearson correlation coefficient, marked concordance between expression and methylation was observed. Of 490 hypomethylated genes in cell lines growing in the brain, expression of 77.2% of genes showed significant correlation ($P < 0.05$). In particular, the majority of genes (59.4%) showed an inverse correlation (Fig. 2*B*), indicating that up-regulation of genes in cancer cell lines growing in the brain might be caused, in part, by epigenetic changes in gene promoters. In contrast, although a similar number of promoters were hypermethylated in tumors growing in the brain, these promoters were weakly associated with changes in expression, with 33.4% of genes showing an inverse correlation of expression with methylation (Fig. 2*C*).

The concordance of gene expression and methylation patterns in tumor cells growing in the brain resembles cell fate determination during development (13), and furthermore, transcriptional programs of neuronal progenitor cells are changed dramatically by transcription factors that respond to both intrinsic and extrinsic factors (14). Thus, we sought to find potential key transcriptional signaling networks that might drive the dramatic reprogramming of the transcriptome of tumor cells growing in the brain. Of 405 genes annotated as transcription factors in the gene ontology database, we identified 65 genes as brain-specific transcription factors with expression that is significantly higher ($P < 0.001$ by two-sample t test) (Fig. S6) in brain tissues using the mouse gene expression data from the normal brain. Among these genes, expression of 20 transcription factors was significantly ($P < 0.001$ by two-sample t test) up-regulated in the human tumor cells xenografted in brain when transcriptional profiles from human gene expression data were analyzed independently (Fig. 3*A*).

Because epigenetic changes in regulatory regions of transcription factors are key mechanisms for activation of fate-determining genes (15, 16), we cross-compared expression of these genes with methylation data. Among the brain-specific transcription factors, the expression of six transcription factors (*PURB*, *ONECUT2*, *ESRRG*, *NFIB*, *TCF4*, and *MEF2C*) was significantly ($P < 0.001$) associated with methylation of their promoters. Specifically, expression of *PURB*, *ONECUT2*, *ESRRG*, and *TCF4* was negatively correlated with methylation, indicating that they might be part of the core transcriptional signaling network responsible for reprogramming the cancer transcriptome in brain metastases (Fig. 3 *B* and *C*). Interestingly, *ESRRG* and *TCF4* have been implicated in differentiation of neuronal progenitors and neuronal cell survival (17).

To validate epigenetic changes identified from methylation microarray experiments, we carried out methylation-specific PCR (MSP) experiments on CpG islands in promoter regions of human *ESRRG*, *CCL27*, and *SPI1* genes with genomic DNA from xenografted tumors. Although most of the CpG islands were highly methylated in fat pad-xenografted tumors, a significant proportion of the CpG islands was demethylated in brain-

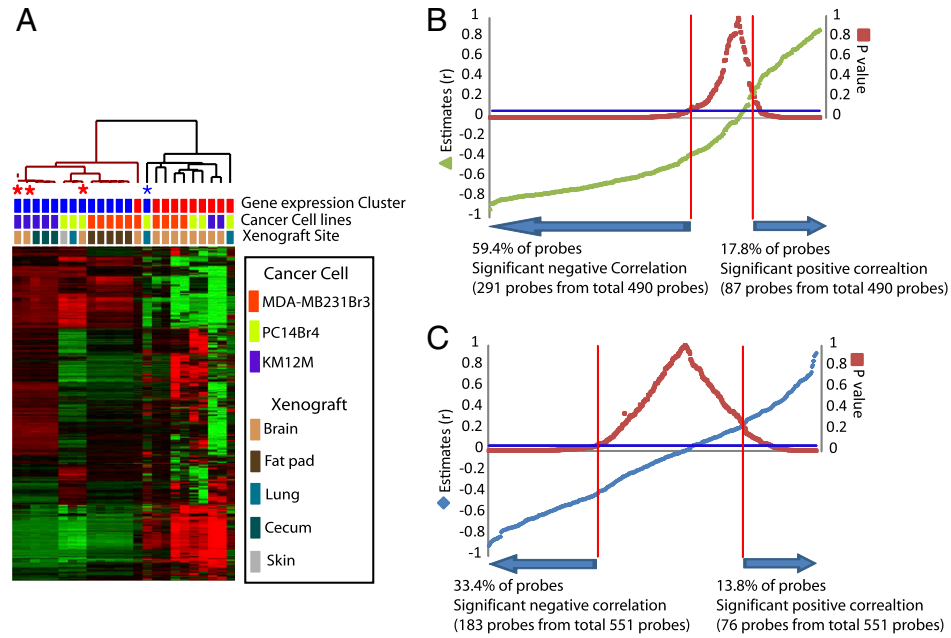

Fig. 2. Epigenetic changes driven by brain microenvironment. (*A*) Unsupervised clustering of methylation profiles in xenografted tumors. The methylation data showing the top 25% of variance were used for this analysis. The clustering analysis was performed after median centering of methylation data across tissues in each cancer cell types. In the tissue identification code row of the dendrogram, three brain-xenografted tissues (two KM12M and one PC14Br4 tissues) grouped with orthotopically xenografted tissues in gene expression clusters are highlighted in red, and an orthotopically xenografted PC14Br4 sample grouped with brain-xenografted tissues in gene expression clusters is highlighted in blue. (*B*) Pearson correlation coefficient calculation of the genes showing hypomethylation in brain-xenografted tumors. (*C*) Pearson correlation coefficient calculation of the genes showing hypermethylation in brain-xenografted tumors.

xenografted tumors, which was evidenced by differences in MSP products (Fig. S7*A*). To ensure that the CpG islands examined in microarrays and MSP reside in human promoters and not mouse promoters, we carried out bisulfite sequencing of human promoter regions of these genes. In consistent with previous analyses, CpG islands in three genes were significantly demethylated in brain-xenografted tumors (Fig. S7*B*), strongly supporting that demethylation in human CpG islands of promoter regions is accountable for significant negative correlation (*CCL27*, $r = -0.855$; *ESRRG*, $r = -0.833$; *SPI1*, $r = -0.903$; $P < 1.0 \times 10^{-5}$ for all three genes) between methylation status and expression of the three genes observed from microarray experiments (Fig. S8).

Transcriptome Reprogramming Is Partially Induced by Coculture of Cancer Cells with Astrocytes. Because extrinsic factors such as cytokines and cell to cell interaction are key mediators for cell fate determination during development (18), we hypothesize that cellular stimulation that mimics the microenvironment of the brain could induce the changes in transcriptomes observed in cancer cells growing in the brain microenvironment. To test this hypothesis, MDA-MB-231 cells were cocultured with mouse astrocytes to mimic the microenvironment of brain or fibroblast NIH 3T3 cells as control. Gene expression data from each coculture were collected independently using cross-species hybridization with human and mouse microarrays. Interestingly, expression of a large number of genes (2,946 genes) was significantly ($P < 0.001$ by two-sample t test) altered when MDA-MB-231 was cultured with astrocytes but not with fibroblasts. Moreover, the overall gene expression patterns of cancer cells cocultured with astrocytes were remarkably similar to those patterns from MDA-MB-231 growing in the brain, strongly indicating that interactions with astrocytes might contribute to the reprogramming of the cancer cell transcriptome (Fig. 4). Although the patterns of changes were similar, the magnitude of the effect was less with cultured astrocytes compared with the cancer cells growing in the brain microenvironment, suggesting that additional factors in the brain microenvironment participate in full transcriptome reprogramming. To further test species specificity of probes under our hybridization, we carried out additional microarray experiments with GFP-expressing MDA-MB-231Br3 cells that were FACS sorted after coculturing mouse astrocytes or NIH 3T3 cells. Gene expression patterns of FACS-sorted cancer cells that are almost free of mouse astrocytes were highly similar to those patterns from directly harvested cancer cells from coculture (Fig. S9). These data again strongly suggested that our experimental condition for CHME was well-optimized for detecting species-specific gene expression data from mixed RNAs.

Discussion

The molecular and cellular characteristics of cancer cells metastatic to particular organs remain unclear because of, in part, the lack of experimental approaches that can characterize metastatic tumor cells and the microenvironment at the molecular level. In this study, we showed that an experimental strategy, CHME, can characterize the transcriptome of cancer cells in metastatic sites by discriminating gene expression signatures of cancer cells from host cells in a single xenograft tissue. This approach allowed us to uncover unexpected characteristics of cancer cells in a brain metastasis mouse model, showing that the tumor cells acquire expression patterns found in neuronal cells.

Several lines of evidence indicate that the microarray platform and experimental conditions applied achieved sufficient sensitivity and specificity to distinguish signals of tumor cells from stroma cells and vice versa. First, microarray experiments with mixtures of human and mouse RNA showed that hybridization conditions can discriminate foreign species of RNA even when they constitute 90% of the RNA mixture and can detect expression of species-specific genes even when they only represent 10% of the mRNA mixture (Fig. S1). Second, the global gene expression pattern of the RNA mixtures (mouse brain and PC14Br4 cell line from tissue culture) was highly similar to

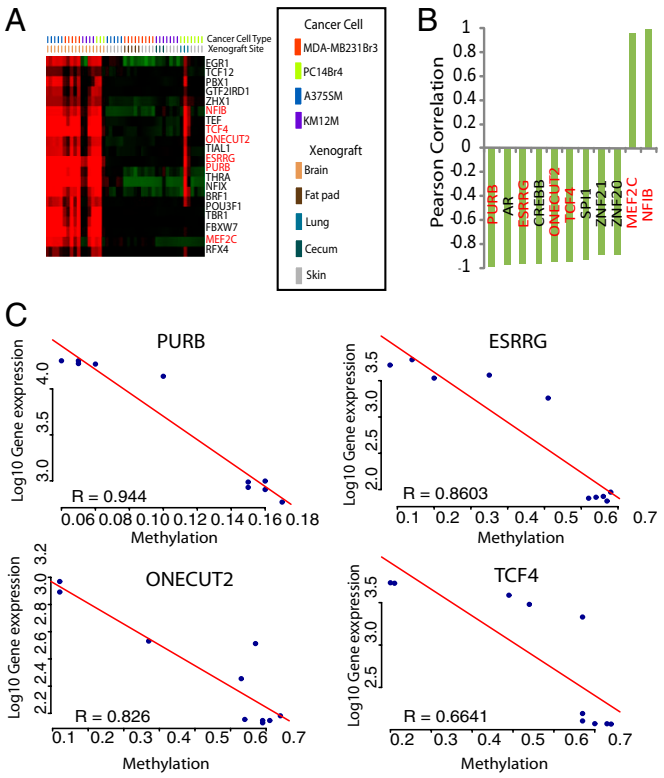

Fig. 3. Brain-specific transcription factor. (*A*) Heat map of brain-specific transcription factors in human gene expression profiles; 20 transcription factors that were shown brain-specific expression in mouse array data (Fig. S6) and significant increases in brain-xenografted tumors in human array data ($P < 0.001$) at the same time were selected for the presentation of their expression. (*B*) The transcription factors showing significant correlation between methylation and expression in MDA-MB-231Br3 cells were xenografted in brain and fat pad; 11 transcription factors showing significant increase in its expression in brain-xenografted tumors ($P < 0.001$ and more than threefold) with significant correlation with methylation status ($P < 0.001$) were presented. (*C*) Scatter plot of four brain-specific transcription factors that are showing significant negative correlation between gene expression and methylation status.

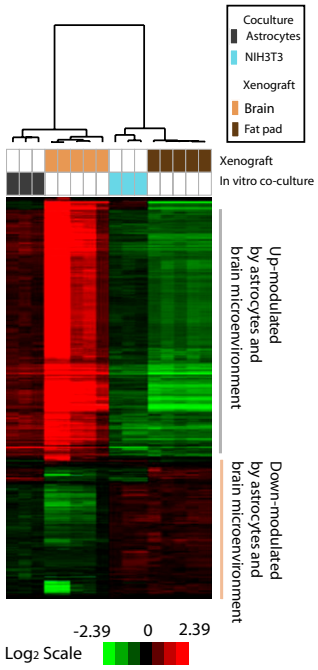

Fig. 4. Comparison of in vitro coculture signature with in vivo brain-xenografted signature. Expression pattern of 1,085 genes with expression that is significantly ($P < 0.001$ by two-sample *t* test) altered by both coculture with mouse astrocytes and brain microenvironment. Before clustering, gene expression data were median centered independently.

PC14Br4 xenografted to lung or skin when they were hybridized with human microarrays but was highly similar to brain tissue when they were hybridized to mouse microarrays (Fig. 1). Third, IHC staining of FFPE tissues clearly showed that human cancer cells in brain-xenografted tissues exclusively express brain xenograft-specific genes (Fig. S3). Fourth, gene expression patterns of FACS-sorted (free of mouse astrocytes) cancer cells from cocultures are highly similar to those patterns of cancer cells directly harvested from coculture (Fig. S9). Our data show that cancer cell-specific gene expression patterns can be clearly elucidated in a tumor mass consisting of a mixture of cancer cells and nontumor host cells without extensive cell manipulation or microdissection.

Transcriptome reprogramming of the cancer cells growing in tumors resembles the fate determination of neuronal stem cells mediated by dynamic interplay of extrinsic factors, such as environmental signals and cell to cell interactions, and intrinsic factors, such as epigenetic and transcriptional events (15, 16). Coculture of cancer cells (MDA-MB-231) with mouse astrocytes, mimicking the microenvironment in brain, induced transcriptome reprogramming, indicating that interactions with astrocytes might contribute extrinsic factors triggering intrinsic transcriptional events. Our previous study showed that physical interaction between astrocytes and cancer cells is necessary for induction of gene expression (19). Although selective genetic events in the brain microenvironment might contribute to reprogramming of the tumor transcriptome, the high concordance between promoter methylation profile and gene expression profiles strongly suggests that epigenetic events contribute to transcriptome reprogramming. Four transcription factors (*PURB*, *ONECUT2*, *ESRRG*, and *TCF4*) were markedly induced in tumor cells growing in the brain and showed a significant negative correlation between methylation status and gene expression status. *TCF4* has previously been implicated in neuronal cell differentiation and maintenance, suggesting that it may be a contributor (14).

In breast cancer, clinically symptomatic metastases to the brain occurs in 10–15% of patients who develop metastatic breast cancer (20). Unfortunately, brain metastases shows a poor response to chemotherapeutic agents that are active in the primary tumor site (21). The unexpected characteristics of tumor cells growing in the brain elucidated by our experimental approach may help to explain the mechanisms underlying the chemoresistance of these tumors. Malignant glioblastoma, the most aggressive type of brain tumor, is highly resistant to chemotherapy (22, 23). In addition to the existence of a blood–brain barrier, which potentially limits the delivery of therapeutic agents to the metastatic tumor, the lack of response to chemotherapy could be because of, in part, the intrinsic resistance of cancer cells that is an inherent characteristic of neuronal lineage cells. When xenografted in the brain, all four cell lines tested in this study acquired neuronal expression patterns that can also be induced by culture with astrocytes (Fig. 4). Taken together, our data suggest that acquisition of neuronal transcriptional patterns in cancer cells might contribute to the chemoresistance nature of brain metastasis tumors. Although the strong correlation between epigenetic changes with transcriptome reprogramming is compatible with methylation playing a role in the transcriptional changes, the key events that trigger the transcriptome reprogramming remain to be fully elucidated.

Materials and Methods

For application of CHME strategy to in vivo metastasis models, human cancer cells were introduced into various organ sites, including brain, skin, fat pad, cecum, and lung, in immunocompromised mice to mimic the in vivo microenvironment of different sites of metastasis. Gene expression data from human and mouse microarrays were independently collected from harvested tumor masses. Detailed methods for all experiments and statistical analyses are provided in *SI Materials and Methods*.

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